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Histochemical Measurements of Rat Kidney Hexokinase[1] (38616)

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(Introduced by P. L. McLain)

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Studies on the development of the kidney nephron have focused upon histological and functional aspects and less upon the quantitative enzymatic changes. During ontogenesis of the nephron, there is a remarkable anatomical and functional transformation in which the kidney evolves its complex endocrine and excretory character. It is believed that this character can be better understood if enzymes in substructures of the nephron are quantitated in both the developing and adult stages.

Hexokinase (ATP: D-hexose phosphotransferase, E.C.2.7.1.1) (HK) catalyzes the ATP-dependent phosphorylation of glucose yielding glucose-6-P (2). Three of the four known HK isozymes are found in the adult kidney (3). Functionally, this enzyme catalyzes a rate limiting step in glycolysis. In addition, it is believed that glucose reabsorption in the kidney is associated with an early phosphorylation of glucose by hexokinase (4). Previous measurements in the adult kidney reveal significant differences between cortex and medulla and in certain substructures of the nephron (5–7). However, even though specific activity in the whole kidney is at the adult level by birth, the relative level in cortex and medulla does not attain the adult pattern until 21 days of age (5, 8). In order to elucidate further these changes which occur during development, the quantitative measurement of HK in different portions of the nephron follows.

Materials and Methods. The experiments were carried out on kidneys obtained from the litters of timed pregnant Sprague–Dawley albino rats (Charles River, C. D-Strain). Fetuses were obtained from mother rats anesthetized with ether. They were immediately plunged into Freon-12 chilled to $-150°$ with liquid N_2. Newborn animals (less than 12 hr old) were frozen whole in the Freon. Older animals were male only and were injected intraperitoneally with 20 mg/kg trypan blue 48 hr before sacrifice to facilitate structural identification (9). The rats were anesthetized lightly with ether and the kidneys were frozen *in situ* with Freon. The kidneys were cut into 25 μm frozen sections at $-20°$, lyophilized at $-35°$ then stored at $-90°$ *in vacuo* until dissected. The general quantitative histochemical procedures for dissection, weighing on a quartz fiber balance and assay have been described in detail elsewhere (10, 11).

Analyses were made on whole kidneys and on histologically defined samples dissected from frozen dried sections. Larger dissected samples (about 1 μg dry wt) consisted of patches from either the neogenic zone (early ages only), outer cortex, inner cortex, outer medulla, medullary ray or inner medulla (includes papilla). The smaller samples (5–50 ng dry wt, balance sensitivity 0.05 μg/mm) consisted of specific segments from individual nephrons. With the aid of the trypan blue intravital staining procedure, and consideration of obvious structural characteristics, it was possible to identify glomeruli (G), most of the discrete portions of the nephron as well as small arteries. Histochemical staining *in vitro* was carried out to gain familiarity with the trypan blue *in situ* stained structures but *in vitro* staining was not necessary for identification of samples taken for analysis. The proximal convoluted tubules (PCT) were stained blue by trypan blue. The unstained pars recti tubules (PRT)

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were readily identified in the inner cortex. The thin descending limb of Henle could be identified, but assays gave extremely variable results. The ascending limb of Henle (ALH) was identified as a gray–bluish tubular structure predominantly in the outer medulla. The distal convoluted tubules (DCT) were unstained and found in proximity to glomeruli. Collecting tubules (CT) were taken from medullary rays in the outer medulla. The developing rat kidney cortex is a predominantly neogenic zone in which new glomeruli and tubules form. This zone is present up to about 10 days of age (9, 12). The glomeruli were easily identified but the tubules of this zone could not be subdivided into the various substructures found in more developed kidney cortex.

Characterization of HK for the histochemical assay was performed on homogenates of fresh or frozen kidney prepared in ground glass containers at 0° utilizing 8–10 vol of 100 m*M* Tris buffer (pH 7.6), unless otherwise indicated. HK was assayed at 25° in 1 ml of reagent containing kidney (1:20,000 tissue dilution), 5 m*M* glucose, 6 m*M* ATP, 1 μg/ml glucose-6-P dehydrogenase, 3 μg/ml 6-P-gluconic acid dehydrogenase, 5 m*M* $MgCl_2$, 0.5% Triton X-100, 0.05% bovine serum albumin, 1.2 m*M* NADP+, and 100 m*M* Tris·HCl, pH 7.6. Reactions were allowed to proceed for 10–60 min. Fluorescence was measured directly, or aliquots were diluted and assayed by direct fluorescence in 0.02 *M* phosphate buffer, pH 7.6. Enzyme activity is expressed as moles glucose phosphorylated per kilogram tissue (wet or dry) per hour at 37°, (*M* kg H). There was sufficient lactonase in the tissue to effect complete transformation of 6-P-gluconolactone to 6-P-gluconic acid within 3 minutes so that lactonase was not added to the reagent.

Histochemical analysis was carried out by the oil well technique with the reagent covered with 40% hexadecane: 60% mineral oil to prevent evaporation (11). Samples (5–50 ng) were pushed through the oil at timed intervals into 1 μl HK reagent. Reactions were allowed to proceed for 30–60 min at 25°, then 2.5 μl of 0.3 *M* Na_3PO_4–0.3 *M* K_2HPO_4 was added to stop the reaction. Each set of 45–60 samples in the Teflon oil well was then placed on a sand bath at 70° for 15 min to destroy unreacted NADP+. The mixture was cooled and 3 μl was transferred to 1 ml of 6 *N* NaOH containing 0.03% H_2O_2. Fluorescence was induced by heating at 60° for 10 min and read directly. The tubes were protected from bright light. This indirect fluorescent measurement of NADPH gives a six- to eightfold increase in sensitivity (10). Analyses on 1 μg samples were carried out similarly, but in 10 μl reaction volumes.

Electrophoresis of HK isozymes was carried out on polyacetate strips (sepraphore III) with slight modification of a previously published method (13). Kidney was homogenized in an equal volume of ice cold 100 m*M* Tris·HCl, pH 8.0, 10 m*M* glucose, 5 m*M* EDTA and 5 m*M* mercaptoethanol. The homogenate was centrifuged at 100,000 *g* for 1 hr. The supernatant, 10–14 μl, was applied to the polyacetate strips. Electrophoresis was carried out at 4° for 90 min at 0.5 mA/cm. The strips were stained at 37° for 20 min in the standard solution utilizing 0.5 and 100 m*M* glucose. The strips were washed 1 min in methanol–glacial acetic acid–water, 5:1:5 and dried. To make the strips transparent soaking in absolute methanol for 2 min was followed by 2 min in methanol–glacial acetic acid 20:1, then the strips were allowed to dry between two glass plates.

Results. Enzyme characteristics. Characteristics of newborn and adult rat kidney HK are presented in Table I. The K_m for ATP and glucose, linearity and temperature effects were similar for newborn and adult. The pH optimum was lower for newborn in that activity fell off above pH 8 but was plateaued in the adult. Subsequent reactions were carried out at pH 7.6 since this was near optimal for newborn and adult. HK in newborn and adult with 100 m*M*, rather than 5 m*M* glucose, revealed no increase in activity. This does not completely rule out a Type IV HK which has a high K_m for glucose, but it was concluded that in regard to kinetic data, the adult and newborn were sufficiently similar to allow histochemical analysis.

TABLE I. HEXOKINASE CHARACTERISTICS IN WHOLE KIDNEY HOMOGENATE.

	Newborn	44-day adult
Michaelis—Menten constant		
ATP	0.7 m*M*	0.7 m*M*
Glucose	0.04 m*M*	0.04 m*M*
pH Optimum	7–8	7.5–10
Linearity with time	0–60 min	0–60 min
Linearity with tissue (mg/ml)	0.01–1.5	0.01–1.5
Temperature $V_{37°}/V_{23°}$	2.18	2.32

Electrophoresis was carried out to compare further the newborn and adult enzyme. The kidneys from nine newborn rats were combined before preparation of the 100,000 *g* supernatant and kidneys from two adult rats were utilized. Adult rat liver served as the reference. Adult liver had isozyme bands at 0.3, 1.5, 2.0 and 2.8 cm (compatible with Type I, II, III and IV isozymes). Adult and newborn kidney revealed bands at 0.3 and 1.3 cm corresponding to Type I and II isozymes. There was questionable staining at the Type III region. In addition, newborn exhibited very heavy staining at the origin not seen in adult kidney and liver. This origin staining was decreased, but not eliminated in the absence of ATP. The finding of predominately Type I and II in kidney is compatible with other studies (14). But a fetal enzyme with very low electrophoretic mobility has not been ruled out.

The effect of trypan blue was studied by comparing whole kidney HK activity (wet wt) to that in noninjected animals: Control 0.94 ± 0.09, $n = 6$; trypan 0.84 ± 0.11, $n = 6$. The body weights also did not differ; control 222 ± 7, $n = 6$, trypan 226 ± 14, $n = 6$.

HK was not affected by the freeze drying process. No activity was lost after storage in the freeze dried state for 2 yr, but whole tissue homogenate gradually lost activity in the frozen state.

Histochemical measurements. Quantitative histochemical measurements in various zones of dried kidney sections are shown in Table II. Activity was highest at 8 days of age with medulla being the most active. When activity in the neogenic zone of 4-day animals is compared to its outer cortex, a pattern consistent with activity increasing with maturation is observed since the neogenic zone is where new glomeruli and tubules begin to form. However, in all other zones activities decrease with maturation to 22 days. By 44 days the activity in the outer medulla assumes an intermediate position between the 8- and 22-day age. The outer medulla has a heavy concentration of ALH. Other studies on frozen kidney which was sectioned serially from cortex to papilla have also revealed similar levels of activity and that activity decreased with maturation exhibiting a biphasic pattern in the outer medullary region (5). In the present study, measurements on zones of newborn and prenatal kidney were not carried out because of inadequate differentiation for identification.

HK in the immature neogenic zone G and tubules is presented in Table III. Activity in G of −4 day, newborn and 4-day-old kidney is approximately the same. However, in this time span the activity in the cortical tubules fell to about half. The cross-sectional area of the kidney reveals that by 8 days less than 5% of the cortex is made of neogenic zone, whereas in the newborn the entire cortex is neogenic zone (9, 12). When tubules of newborn are taken from the central portion (medullary) of the kidney, Table III, activity is 1.6-fold higher, ($P < .05$) indicating significant differences of enzyme distribution within the nephron at a very early age.

Activities in samples from older nephron are presented in Table IV. Values from G decrease with development. Activity in PCT is lowest in the 44-day kidney. The measurements in PRT reveal an upward trend of activity by 44 days, although statistically insignificant. The 44-day values for G, PCT and PRT are similar to previously reported

TABLE II. HEXOKINASE ACTIVITY IN ZONES OF DEVELOPING RAT KIDNEY.[a]

Zone	Age (days) 4	8	22	44
Cortex				
Neogenic	1.68 (0.21) 13	—	—	—
Outer	3.27 (0.51) 16	2.85 (0.59) 10	1.02 (0.15) 13	0.78 (0.24) 16
Inner	—	2.47 (0.32) 10	0.92 (0.12) 14	1.06 (0.19) 20
Medullary ray	—	2.80 (0.29) 14	0.55 (0.07) 14	1.17 (0.27) 19
Medulla				
Outer	—	3.93 (0.36) 14	1.10 (0.23) 11	2.47 (0.40) 18
Inner	—	4.32 (0.86) 13	0.88 (0.14) 13	1.05 (0.24) 19

[a] Activities were measured in 0.2–1 μg tissue sections taken from the designated zones and are expressed as moles glucose phosphorylated per kilogram dry tissue per hour at 25°. The average activity and the standard errors in parentheses were calculated as though all the data came from a single animal. Below is the number of histological samples analyzed. There are three animals in age groups 4, 8 and 22 and four rats in age group 44 days.

TABLE III. HEXOKINASE ACTIVITY IN NEOGENIC ZONE OF RAT KIDNEY.[a]

	Age (days) −4	Newborn	4
Glomerulus	1.33 (0.20) 11	1.50 (0.30) 12	1.37 (0.26) 13
Cortical tubules	2.25 (0.35) 14	1.78 (0.37) 14	1.18 (0.15) 16
Medullary tubules	—	2.78 (0.27) 13	—

[a] Activities were determined on 5–50 ng tissue and are expressed as in Table II. There are three animals in each group.

studies in young adult rats (6, 7). From 8 to 44 days, the activity increased threefold in the ALH. However, the ratio of activity in the ALH compared to PCT is about one at 8 days, but increases to five by the 44 day stage. This ratio in the adult has been noted previously (7). The adult pattern in DCT is attained by 8 days of age. The CT as well as the G and PCT reveals a marked rise in activity in the 22-day age but then decreased by 44 days. This was due to large variation between animals, but no consistent pattern is evident. For example in PCT the average activity in one 22-day animal was 4.04 MKgH, but it was 0.51 and 0.70 in two others of the same litter (not shown). But in PRT it was 1.69, 1.10 and 0.75 respectively. The variability could not be attributed to experimental error since repeat assays were reproducible. In addition, other studies on homogenates of developing kidney revealed marked fluctuations in the 14- to 22-day age groups (5). Small arteries, which were not part of the nephron, exhibited a reduction in activity with maturation of the nephron.

Discussion. The present study demonstrates measurable levels of HK in substructures of the developing and adult kidney nephron. The activities were directionally

TABLE IV. HEXOKINASE ACTIVITY IN DEVELOPING RAT KIDNEY NEPHRON.[a]

	Age (days)			
	4	8	22	44
Glomerulus	1.65 (0.53)	1.05 (0.14)	1.91 (0.44)	0.69 (0.06)
	11	17	10	35
Proximal	1.33 (0.16)	0.96 (0.14)	1.75 (0.83)	0.64 (0.08)
	15	14	8	26
Pars recti	—	0.85 (0.15)	1.03 (0.19)	1.06 (0.16)
		15	13	19
Ascending	—	1.08 (0.15)	1.60 (0.28)	2.93 (0.46)
		16	12	19
Distal	—	0.94 (0.14)	1.19 (0.23)	1.13 (0.22)
		12	10	12
Collecting	—	0.71 (0.06)	2.15 (0.41)	0.99 (0.09)
		15	14	12
Small artery	—	1.88 (0.44)	1.18 (0.38)	0.77 (0.13)
		14	10	18

[a] Activities were determined on 5–50 ng tissue and are expressed as in Table II. There are three animals in groups 4, 8 and 22 and four animals in age group 44 days.

different from that which have been reported in whole homogenates. There was a twofold decrease in G and PCT HK activity while activity in ALH increased threefold. These changes were not necessarily linearly age dependent in a uniform manner as seen in Tables II-IV. Whether this variability represents interplay with other concomitantly developing enzyme systems, hormonal influences, or decreasing levels of maternally synthesized and placentally transferred factors influencing relative synthesis in young animals is purely speculative.

Data on the *in vitro* level of a single or group of enzymes does not necessarily establish a quantitative or qualitative function since the local kinetic parameters actually function *in vivo* may greatly distort assessment by *in vitro* techniques of the *in vivo* activity. However, the methods utilized in the present quantitative histochemical analysis allow the enzymatic reaction to be quantitated at a high tissue dilution under standardized conditions. This provides a quantitative approximation of the total *in vivo* enzyme concentration that could be available in substructures of the nephron. This type of data usually cannot be obtained from qualitative histochemical staining procedures on tissue slices because of local factors and the difficulty of quantitating the colors of the dyes.

The present studies of HK distribution in the developing nephron are consistent with findings reported by other investigators. Enzymatic analysis on homogenates of whole kidney taken from animals in various developmental stages have revealed that HK and other enzymes of glycolysis undergo little change, but that gluconeogenic enzymes may increase 20-fold (8). In an earlier study it was found that in renal cortex, particularly in the outermost portion, HK falls during maturation (5). Conversely, in the papilla an increase in HK was observed with age. This latter study, like the present report, reveals that analyses on anatomical subdivisions of kidney will provide developmental patterns which differ from that observed in whole kidney. The data on substructures of the nephron reveal that if HK can be quantitatively related to function, then glycolysis is relatively more active in neogenic zone structures and in adult ALH.

The measurement of HK was carried out because this enzyme may serve several functions. Furthermore, it is believed that these functions can be better understood if the enzyme is quantitated in substructures of the nephron throughout development. Not only is HK necessary to commit glucose to energy production and nourishment of the developing tissue, but through transtubular transport it may regulate optimal carbo-

hydrate stores by eliminating spillage of glucose into the urine in the normal state and by modifying plasma glucose concentrations in states of hyperglycemia.

The mechanism of glucose reabsorption by the kidney is unsettled (4, 15). Various physiological studies have concluded that glucose present in the glomerular filtrate is completely reabsorbed in the PCT. The initial binding of glucose to the renal proximal tubule luminal membranes is stereospecific and apparently occurs without phosphorylation (4). What happens beyond this step is controversial. Evidence was presented that the enzyme trehalase, which hydrolytically splits the disaccharide trehalose to two glucose moieties, functions in the active transport of glucose from the glomerular filtrate and across the intestinal mucosa (16). In this scheme, glucose from the glomerular filtrate is first phosphorylated by HK. Subsequently in this scheme are the enzymes phosphoglucomutase, UDPG-pyrophosphorylase, trehalose-6-P synthetase, trehalose-6-phosphatase and trehalase. The initial substrate is glucose and the final product of the reaction is glucose. The localization of disaccharidases in the canine kidney have been assessed by an *in vivo* multiple indicator dilution technique (15). The evidence was compatible with the disaccharidases located in the glycocalyx region of the brush border and spatially superficial to the glucose transport system.

The present quantitative histochemical studies do not necessarily support the concept of a disaccharidase system mediating glucose reabsorption. Single superficial nephron glomerular function increased progressively until the adult stage of development (17, 18). But G and PCT HK decreased by half, even in the neogenic zone, under circumstances in which nephron function was developing. At the same time HK increased threefold in ALH, a nephron substructure where glucose reabsorption apparently does not occur. UDPG-pyrophosphorylase which is a central enzyme in the trehalase postulate was noted to increase most dramatically in PCT, PRT and ALH during development (19). However, a second enzyme UDPG-pyrophosphatase, which degrades UDPG, was progressively higher in PCT and PRT and very slow in ALH. These measurements taken with those on HK are most consistent with the concept that HK is required for trapping the glucose for metabolism, and that this is separate from glucose reabsorption.

Summary. HK has been measured in substructures of the developing rat nephron. Reactions were carried on samples dissected from freeze dried kidney and assayed in 1 μl reaction volume utilizing the "oil-well" technique. Samples from 4 days prenatal to 44-day adult were analyzed. Activity during development decreased in G, PCT, and it increased in ALH. There was little change in PRT, DCT and CT. Activity in small arteries decreased.

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